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# Postnatal development of the optic nerve in (C57BL × CBA)F1 hybrid mice: general changes in morphometric parameters

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## ABSTRACT

A morphometric analysis of the optic nerve in different age groups of (C57BL × CBA)F1 hybrid mice was carried out. Morphometric parameters examined were mean nerve cross-sectional area (*csa*), mean myelinated nerve fibre count, mean myelinated nerve fibre density and myelinated nerve fibre size distribution. The findings revealed that the optic nerve continues to develop well into adult life. Growth in calibre was very rapid during the early stage of postnatal life, but progressively slowed with age thereafter. No myelinated nerve fibres were observed before the 5th day of postnatal life. Similarly, once myelination was initiated, it progressed very rapidly during the early stage of postnatal development and, as for the *csa*, it slowed thereafter. Peak level of myelination within the optic nerve, which corresponded with the age when the maximum number of myelinated nerve fibres, i.e.  $94213 \pm 1799$  (S.E.M.) was measured, occurred at the 16th week of postnatal life. The earliest myelinated nerve fibres seen were predominantly large in diameter, but with increasing age, fibres of smaller diameter dominated the myelinated nerve fibre spectrum in the nerve. The highest mean myelinated nerve fibre density was observed in mice at the age of peak myelination.

*Key words:* Growth; myelination.

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## INTRODUCTION

Many studies have been carried out to analyse the postnatal development of the optic nerve both in man (Sylvester & Ari, 1961; Dolman et al. 1980; Balazsi et al. 1984; Repka & Quigley, 1989; Day, 1990), and in the rat (Skoff et al. 1976*a, b*; Tennekoon et al. 1977; Lam et al. 1982; Perry et al. 1983). The majority of studies undertaken with mice have been concerned with the prenatal morphogenesis of the eye, rather than with the detailed analysis of its component parts, such as the optic nerve (Truslove, 1962; Pei & Rhodin, 1970; Robb et al. 1978; Theiler et al. 1980; Theiler & Varnum, 1981; Franz & Besecke, 1991).

Both Gyllenstein & Malmfors (1963) and Gyllenstein et al. (1966) studied the postnatal development of the optic nerve in C57BL mice, although these reports were not primarily concerned with the normal postnatal development of the optic nerve in this strain.

Furthermore, they analysed only a few age groups of mice. In their controls, Gyllenstein & Malmfors (1963) observed only unmyelinated fibres at day (d) 5, after which time myelination progressed at a slow rate until d 15, when it progressed rapidly up to d 20. Little increase in myelination was then observed up to d 30, after which time there was no further increase in the number of myelinated nerve fibres present. The majority of myelinated fibres had a diameter of less than 1  $\mu\text{m}$ . No unmyelinated fibres were observed in their adult mice. In a subsequent control study, Gyllenstein et al. (1966) observed a significant increase in the *csa* and total number of myelinated nerve fibres present between 2 and 4 mo of age, followed by a slight decrease in both parameters between 4 and 7 mo. Growth of the nerve continued well into adulthood.

The findings from other species have been quite variable. Sylvester & Ari (1961) demonstrated that the

human optic nerve increases maximally in size during the 1st year and continues to do so, though with diminished velocity, up to the 4th year. These findings are in general agreement with those of Todd et al. (1940). Dolman et al. (1980; see also Balazsi et al. 1984) also reported a marked decrease in the rate of growth of the optic nerve between the 4th and the 12th year, with adult size being attained between the 12th and 15th years.

This report is one of a series of studies on the morphometric analysis of the optic nerve in different strains of mice. It was also undertaken to establish baseline morphometric information necessary for future teratological studies on this strain of mice. Unlike the previous studies (see Dangata et al. 1994, 1995) it has concentrated on the postnatal developmental parameters of the optic nerve in the mouse, as relatively little information is available on this topic in the literature. The optic nerve was obtained from different age groups of both immature and adult F1 hybrid mice and the cross-sectional area (*csa*), total myelinated nerve fibre population, numerical density and fibre size spectrum determined. Information from this study has allowed a comparison to be made between the postnatal features of the optic nerve in the F1 hybrid mouse, and those of other species that have also been studied.

#### MATERIALS AND METHODS

Different age groups of a range of immature and adult male (C57BL  $\times$  CBA)F1 hybrid mice were studied. Five mice each at 2, 3, 5, 9, 16 and 24 wk of age were studied. Each animal was weighed and deeply anaesthetised by an intraperitoneal injection of 0.02 ml/g body weight of 1.2% solution of tribromoethanol (Avertin) in 0.9% saline. In the adult mice, the heart was exposed and, using a 21 G needle, perfusion was carried out by giving 2.0 ml/g body weight of a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer through the left ventricle while the heart was still beating. Immature mice were similarly perfused transcardially, although a 23 G needle was used to avoid rupture of the left ventricle during the perfusion procedure. The surface of the liver was excoriated, to avoid a build-up of fixative in the venous part of the circulation.

The entire length of the optic nerve was immediately and carefully dissected out, avoiding traction on the nerve. Because in 2 earlier studies (Dangata et al. 1994, 1995) no significant difference was observed in any of the parameters to be analysed in the present study between the right and the left optic nerves in

either male or female mice, both optic nerves from each animal were put into the same prelabelled bottle containing the fixative and left for a total of 12 h. The nerves were then washed in buffer and transferred into a secondary fixative consisting of 1% osmium tetroxide in 0.1 M phosphate buffer for another 2 h. After this they were dehydrated through a graded alcohol series and finally embedded in Araldite.

Semithin transverse sections ( $\sim 1 \mu\text{m}$ ) were cut perpendicular to the long axis of each nerve using a Reichert-Jung Ultracut E Microtome. Sections were stained with 1% toluidine blue in 1% borax for light microscopy. For electron microscopy ultrathin sections ( $\sim 80 \text{ nm}$  thickness) were cut. These were picked up on 200 mesh copper grids and subsequently stained with 0.2% lead citrate and a saturated solution of uranyl acetate (Reynolds, 1963). A selection of photomicrographs was taken from the centre to the periphery of each nerve using a Philips EM301 transmission electron microscope. This was done by locating the centre of the nerve (already marked on a sketch for each nerve during photography) and moving in one direction, photomicrographs were randomly taken from the centre, intermediate zone and periphery of the nerve making sure there was no overlap between the fields photographed. The micrographs were developed and printed to a final magnification of  $\times 3000$ .

Using the semithin sections, the cross-sectional area (*csa*) of each optic nerve excluding its meningeal coverings was measured in a Magiscan image analysis system (Applied Imaging). We have used meningeal coverings to describe the extensions of the meningeal sheath that encloses the nerve as described in Gray's Anatomy, namely the dura mater, arachnoid mater and pia mater (Warwick et al. 1989). These were excluded in order to avoid errors that they would introduce to such parameters as the mean nerve fibre count and mean nerve fibre density, since the area occupied by them was devoid of any nerve fibres and the computation of the parameters was directly dependent on the *csa* of the axon-bearing part of the optic nerve. From the *csa* of the individual nerves the mean *csa* of the nerve for each age group of mice was calculated. Other detailed morphometric measurements of the nerves were also carried out on the photomicrographs of the ultrathin sections using the image analyser.

A systematic random sampling method (Mayhew & Sharma, 1984; Mayhew, 1988, 1990) was used to determine the total number and diameter spectrum of the myelinated nerve fibres in each nerve. This was done by locating the centre of the nerve and from this

point sectors of  $10^\circ$  were drawn over different areas of the nerve. A grid of approximately  $1\text{ cm} \times 1\text{ cm}$  squares (equivalent to  $7.8\text{ }\mu\text{m}^2$  of nerve *csa*) was placed over each sector. Using the sampling method indicated above, and starting from the centre of each nerve, every 4th square in each direction (i.e. 1 in 16) was sampled for the estimation of total myelinated nerve fibre counts and fibre diameter (i.e. axon plus myelin sheath) distribution. The objective was to sample between 150 and 200 myelinated nerve fibres, as this enables an estimate of within 95% of the actual number of nerve fibres present in the whole nerve to be obtained. It has been shown that it is not necessary to sample more than 200 nerve fibres for the estimation of the nerve fibre population in the nerve as this would give results that are comparable with those obtained by counting all the individual nerve fibres in the nerve which is uneconomical in terms of time and labour (Mayhew & Sharma, 1984; Mayhew, 1988, 1990). In this method only complete squares falling within each  $10^\circ$  sector were systematically sampled. Also, only myelinated nerve fibres which completely fell within or whose centres fell within a sampled square were included in the analysis. Although the ultrathin sections from each nerve were cut perpendicular to the long axis of the nerve, it was not practicable to cut all the nerve fibres perpendicular to their long axes because of the variable courses they run within the nerve. The external diameter of each sampled nerve fibre (i.e. axon and myelin sheath) was highlighted with the calibrating pen of the image analyser which then automatically measured the shortest diameter which is the true diameter of the highlighted nerve fibre. This resolved the limitation brought about by the variable courses of those fibres not cut along their long axes. The Magiscan image analyser provided a histogram of the sampled nerve fibres in each nerve. From the histograms of the individual nerves in each age group, a summary histogram was plotted for the age group. The number of myelinated nerve fibres present in each whole nerve and their numerical density, i.e. myelinated nerve fibres per  $1000\text{ }\mu\text{m}^2$  was calculated using the ratio technique (Matheson, 1970; Mayhew, 1988, 1990). The mean myelinated nerve fibre count and fibre density of the optic nerve for each age group of mice was also calculated.

In order to determine the approximate age at which myelination in the optic nerve starts, 2 additional mice each at age 9, 8, 7, 6 and 5 d were treated as indicated above. Because of the small number of myelinated fibres present in these samples, the mean myelinated nerve fibre count for these age groups could not be

estimated by the present sampling technique because a total of less than 200 myelinated fibres were present in the selected fields. Additional photomicrographs were therefore taken at a higher magnification and printed to a final magnification of  $\times 66000$  in the 5, 6 and 7 d mice in order to allow the visualisation of any myelin sheaths that could not be seen at the lower magnification.

## RESULTS

### *Cross-sectional areas ( $\mu\text{m}^2$ )*

Growth in the *csa* of the optic nerve was noted to occur from the earliest stage that could be studied using the image analysis system (i.e. age 2 wk) right through into adult life. The increase in the *csa* progressed rapidly early in life up to wk 5 with maximum rate of increase occurring between wk 2 and wk 3. From the end of wk 5 the rate of growth in the *csa* progressively declined with age. The *csa* continued to show an increase even after myelination was completed (see Table 1).

### *Total myelinated nerve fibre counts*

By the postnatal d 5, no clear evidence of myelination was observed from the  $\times 3000$  TEM micrographs, but at  $\times 66000$  it was possible to detect the presence of a few myelin sheaths around some of the largest axons. Increasing evidence of myelination was observed on d 6 and 7 (Fig. 1). Although myelination progressed rapidly once it started, the optic nerve was initially predominantly populated by unmyelinated axons up to wk 2 (Fig. 2). Myelination progressed with such rapidity once it started, that after 5 wk relatively few unmyelinated axons were seen in the nerve. The peak value for the myelinated nerve fibres was seen at 16 wk of age (Fig. 3). Unmyelinated axons were rarely

Table 1. Mean cross-sectional area of optic nerve of different postnatal age groups of (C57BL  $\times$  CBA)F1 hybrids

Age (wk)	Mean cross-sectional area ( $\mu\text{m}^2$ ) ( $\pm$ S.E.M.)*
2	36123 $\pm$ 2319 (10)
3	42560 $\pm$ 3576 (10)
5	50085 $\pm$ 1397 (9)
9	62115 $\pm$ 2322 (10)
16	67107 $\pm$ 1689 (9)
24	71270 $\pm$ 2986 (10)

\* Total analysed in parentheses.

encountered at this age. A decrease in the total number of myelinated nerve fibres counted was, however, observed afterwards (see Table 2).

*Numerical density of myelinated nerve fibres (fibres per 1000  $\mu\text{m}^2$ )*

There was a very rapid increase in numerical density up to wk 5. This was followed by a progressive, but slower increase in mean myelinated nerve fibre density until highest density levels were achieved at an age which corresponded to the time when the maximum number of myelinated nerve fibres was observed in the nerve. A fall in the mean myelinated nerve fibre density was observed after this (see Table 3).

*Myelinated nerve fibre diameter spectrum*

In all age groups no myelinated nerve fibres less than 0.16  $\mu\text{m}$  or greater than 2.0  $\mu\text{m}$  in diameter were measured. The nerve fibre diameter spectrum was unimodal in all age groups. The highest modal diameter of 0.64  $\mu\text{m}$  was observed in the 2 wk age group. There was a general and rapid drop in the modal diameter to 0.40  $\mu\text{m}$  thereafter. The largest mean myelinated nerve fibre diameter, i.e.  $0.67 \pm 0.03 \mu\text{m}$  was observed in the 2 wk old mice. This mean value dropped to its lowest level, i.e.  $0.55 \pm 0.01 \mu\text{m}$  by wk 5. More myelinated fibres at both extremes of the myelinated nerve fibre diameter spectrum were measured with age, and this feature was more marked in those with a larger fibre diameter. For example, the smallest fibres measured were 0.16  $\mu\text{m}$  in diameter, and this was after wk 2, with the largest fibres measured having a diameter of 1.76  $\mu\text{m}$ , and this was observed in the 16 wk age group (see Fig. 4). Beyond wk 3, more than 90% of the myelinated fibres present in the optic nerve had a diameter of less than 1.0  $\mu\text{m}$  (see Table 4).

## DISCUSSION

Morphometric analysis of the optic nerve in the mouse has shown that it continues to develop from birth until well into adult life. Two periods of growth were observed. The initial period of growth was associated with the first evidence of myelination within the nerve, and was characterised by a very rapid increase both in the cross-sectional area and the number of myelinated nerve fibres present in the nerve. This phase of growth lasted up to the end of postnatal wk 5 and was then followed by a second, but progressively slower phase of growth. During the

rapid phase of growth there was approximately a 3-fold increase in the value for mean myelinated nerve fibre count in the nerve at 5 wk over that observed at 2 wk. The maximum rate of growth in *csa* was seen between wk 2 and 3. Our findings on the *csa* of the optic nerve are similar to those of Gyllenstein & Malmfors (1963) with respect to their 2 and 4 mo age groups, although where we observed a gradual increase in growth after 4 mo, they reported a significant decrease in growth at this time. The explanation for this difference has yet to be determined.

The observations in the present study are similar to those earlier reported by Matheson (1970; see also Tennekoon et al. 1977) in the rat, where they also found that the period of maximum growth occurred within the 1st 3 wk of postnatal life. A 2nd period of growth commenced during wk 5 of postnatal life, and lasted well into adult life, although the rate of change in the growth of the nerve during this second period was slower than that observed during the 1st period, and progressively became slower with age. Myelination within the optic nerve was completed during the 2nd period of growth, although the nerve continued to grow in calibre. Hirose & Bass (1973) also reported that in the rat the period during which there is a maximum growth rate occurred between d 10 and d 20 of postnatal life, and paralleled the period of maximum myelination within the nerve. Growth subsequent to d 50 was slow and continued, but at an even slower rate, thereafter. The findings of these authors indicate that there are close similarities between the postnatal development of the optic nerve in the rat and the mouse. In both of these species myelination appears to be an entirely postnatal event (myelination is first seen in the rat on the postnatal d 7; see Vaughn, 1969).

In the human optic nerve, Scammon & Armstrong (1925) reported a slight increase in the diameter of the nerve after birth, and they noted that this principally took place during infancy. Others (e.g. Sylvester & Ari, 1961; Dolman et al. 1980) demonstrated that the human optic nerve is small at birth, but grows very rapidly within the first few years. All, however, indicate that the rate of growth of the human optic nerve slows markedly after an initial relatively rapid growth phase during early life, with the adult size being reached between 12 and 15 y of age (see also Balazsi et al. 1984). The relatively small increase in the size of the human optic nerve after birth suggests that a degree of growth takes place during the prenatal period, for the optic nerve head is almost full size at the time of birth (Day, 1990). The initial postnatal growth

Table 2. Mean myelinated nerve fibre counts in optic nerve of different postnatal age groups of (C57BL  $\times$  CBA)F1 hybrids

Age (wk)	Mean myelinated nerve fibre count ( $\pm$ S.E.M.)*
2	19706 $\pm$ 1474 (10)
3	35107 $\pm$ 3229 (10)
5	68719 $\pm$ 1705 (9)
9	88087 $\pm$ 4896 (10)
16	94213 $\pm$ 1799 (9)
24	77719 $\pm$ 4628 (10)

\* Total analysed in parentheses.

spurt observed in the mouse may correspond to the period of growth from birth to infancy or early childhood reported in man. Again, what appears to be a 2nd and slower period of growth in man lasts only to early adolescence, while in the mouse this 2nd period of growth extends well into adulthood.

The drop in the mean myelinated nerve fibre count observed after the completion of myelination, would seem to suggest that a progressive elimination of myelinated nerve fibres was occurring. Nerve fibres in the middle part of the nerve fibre size spectrum appeared to be maximally affected by this fall in nerve fibre count. This may have been a consequence of a degenerative process in relation to these myelinated nerve fibres, and might explain the rise in the proportion of myelinated nerve fibres at the 2 ends of the nerve size spectrum, as well as a rise in the mean myelinated nerve fibre diameter. This decrease in mean myelinated nerve fibre count with age after the attainment of peak myelination within the optic nerve has also been observed in the C57BL strain of mice (Dangata et al. unpublished results). During normal development an initial over-production of nerve fibres is followed by a period during which degeneration of nerve fibres occurs, so that eventually the adult values are achieved (Potts et al. 1982; Lam et al. 1982; Perry et al. 1983; Crespo et al. 1985; Provis et al. 1985; Repka & Quigley, 1989). The initial period of degeneration coincides approximately with the onset of myelination. In the mouse the reduction in the number of myelinated nerve fibres observed after the period of peak myelination would suggest that by the time peak myelination occurs, the optic nerve still has more myelinated nerve fibres than is required for normal function. This is further explained by the sudden onset of the reduction in number of myelinated nerve fibres after peak myelination. It should not be surprising that the reduction in the total number of myelinated fibres in the optic nerve of the mouse proceeds after the attainment of peak myelination,

since its general postnatal development proceeds well into adult life.

In the human optic nerve, an apparent over-production of axons occurs principally during the first half of intrauterine life, and this is followed by a loss of about 70 % of these axons between 16 and 30 wk of gestation (Provis et al. 1985; Repka & Quigley, 1989) in order to establish adult values. A second phase of degeneration of myelinated nerve fibres in the normal optic nerve occurs significantly later in life, but does not usually occur before the age of 60 y (Dolman et al. 1980). This 2nd phase of degeneration usually corresponds with the onset of old age, and this is in contrast to the situation in the mouse, described in this study. In the mouse, large diameter myelinated nerve fibres characteristically dominate the nerve fibre diameter spectrum early in life. For example, the largest mean myelinated nerve fibre diameter and the largest modal diameter were observed in the 2-wk-old mice. This suggests the presence of a relatively higher proportion of large diameter fibres early in life than is seen during the latter stages of postnatal development. This finding would appear to be similar to that occurring in the human optic nerve at different age groups (Repka & Quigley, 1989). However, after the surge of activities, the reverse phenomenon was observed in the mouse, where the larger diameter fibres were less frequently encountered with increasing age. The diameter spectrum was relatively narrow during the early postnatal period but broadened with age, although fibres with a diameter greater than 2.0  $\mu$ m were not encountered.

This finding is consistent with our previous observations and those of others, that the optic nerve of the mouse is predominantly populated by small diameter nerve fibres (Gyllenstein & Malmfors, 1963; Gyllenstein et al. 1966; Dangata et al. 1994, 1995). The reduction in the mean myelinated nerve fibre diameter observed by the 3rd postnatal week would seem to indicate that by this time myelination predominantly involves the medium size fibres, and that this is maintained until the process is completed. However, the large diameter fibres with which the process started, may continue to acquire additional lamellae of myelin sheath during this period.

Relatively few quantitative studies have been carried out on human optic nerves from different age groups. Those studies that have been undertaken may shed light on why visual function decreases with increasing age in normal individuals (Repka & Quigley, 1989). It is for this reason that appropriate animal models have been used to study both normal and abnormal development of the optic nerve, and



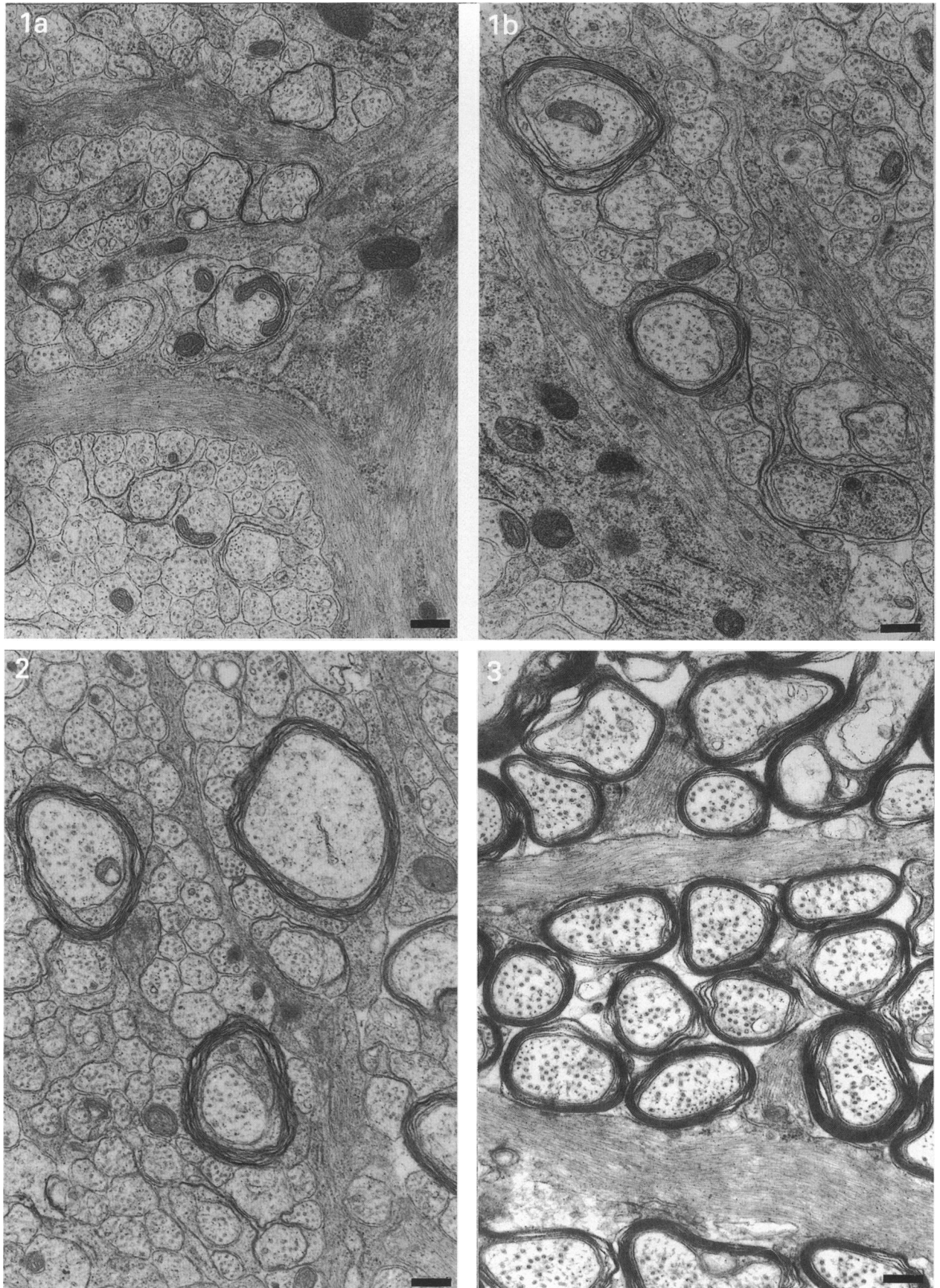


Fig. 1. Representative transmission electron micrographs ( $\times 66000$ ) showing onset of myelination in the optic nerve of (C57BL  $\times$  CBA)F1 hybrid mice during their early postnatal development. (a) Section through the optic nerve of a d 6 F1 hybrid mouse. A few turns of myelin

Table 3. Mean myelinated nerve fibre density in optic nerve of different postnatal age groups of (C57BL × CBA)F1 hybrids

Age (wk)	Mean myelinated nerve fibre density (fibres/1000 $\mu\text{m}^2$ ) (S.E.M.)*
2	546 ± 19 (10)
3	821 ± 22 (10)
5	1378 ± 40 (9)
9	1410 ± 30 (10)
16	1410 ± 41 (9)
24	1087 ± 35 (10)

\* Total analysed in parentheses.

may offer the possibility of identifying specific critical phases during its development (Miller, 1992). The mouse has previously been used to study the toxic effects of substances such as cocaine and alcohol

Table 4. Mean myelinated nerve fibre diameter in optic nerve of different postnatal age groups of (C57BL × CBA)F1 hybrids

Age (wk)	Mean nerve fibre diameter ( $\mu\text{m}$ ) ( $\pm$ S.E.M.)*
2	0.67 ± 0.03 (10)
3	0.59 ± 0.01 (10)
5	0.55 ± 0.01 (9)
9	0.58 ± 0.01 (10)
16	0.57 ± 0.02 (9)
24	0.63 ± 0.02 (10)

\* Total analysed in parentheses.

(Cook et al. 1987; Isenberg et al. 1987). We believe that the mouse is also likely to be a reasonable experimental model to study age-related conditions of

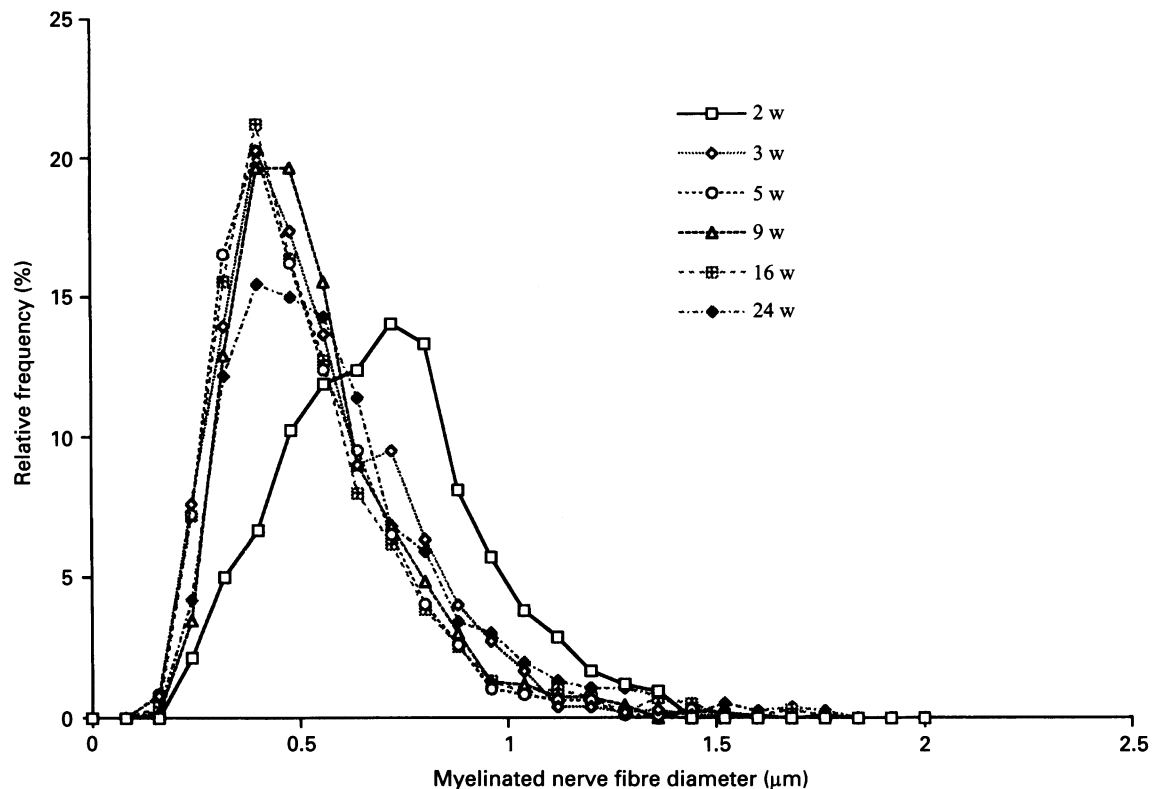


Fig. 4. Histograms showing nerve fibre diameter spectrum in the optic nerve for different postnatal age groups of (C57BL × CBA)F1 hybrid mice. Note the rapid drop in the modal diameter after the 2nd postnatal week to its adult value. The fibre spectrum is unimodal for all age groups and broadens with age. There is a positive skewing of the nerve fibre diameters.

are present around the larger diameter axons. (b) Section through the optic nerve of a d 7 F1 hybrid mouse. While the majority of the axons are still unmyelinated, a few of the larger diameter axons are surrounded by several layers of myelin. Bar, 0.1  $\mu\text{m}$ .

Fig. 2. Representative transmission electron micrograph ( $\times 66000$ ) showing evidence of progressive myelination in the optic nerve of (C57BL × CBA)F1 hybrids by wk 2 of postnatal life. It is mainly the larger axons that have become myelinated. At this stage there is an increase both in the overall diameter of the myelinated nerve fibres and in the number of turns of myelin. Fibres in the middle part of the nerve fibre size spectrum show evidence of early myelination. The optic nerve is still predominantly populated by unmyelinated nerve fibres. Bar, 0.1  $\mu\text{m}$ . See also Figure 4.

Fig. 3. Representative transmission electron micrograph ( $\times 66000$ ) of the optic nerve of an adult (C57BL × CBA)F1 hybrid showing myelinated nerve fibres at the peak level of myelination. Unmyelinated axons are exceptionally rare at this stage. The myelinated nerve fibres present show a broad diameter spectrum. Bar, 0.1  $\mu\text{m}$ . See also Figure 4.



the visual system. Myelination of the human optic nerve begins at about mo 7 of intrauterine life (Scheie & Albert, 1977; Mullaney, 1962), and this approximately corresponds to d 5 of postnatal life in the mouse (Gyllenstein & Malmfors, 1963). While the visual system in man is relatively mature and the eye ready to function within a short period after birth, and remains malleable at least for the 1st decade of life (Mund et al. 1972; Day, 1990), this corresponds approximately to when the surge of myelination activity (wk 2–5) in the postnatal development of the optic nerve in the mouse occurs. It is during the early part of this period that the eyelids reopen (Findlater et al. 1993). By this time, over 50% of the fibres in the optic nerve would have been myelinated. This suggests that this is likely to be the minimum number of myelinated nerve fibres required in the optic nerve to establish normal visual function in the mouse.

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